atomic and ultrafast time resolution combined are lacking, yet will become available. Femtosecond electron diffraction (FED) is a promising tabletop technique with complementary features to XFEL measurements [Miller, R.J.D, Science, 2014, 343, 1108-1116]. It combines the high spatial resolution of diffraction methods with the high temporal resolution of femtosecond optical spectroscopy. The current electron brightness and time-resolution enabled first studies of structural dynamics with atomic resolution on a time-scale of few hundred femtoseconds [Gao, M. et al., Nature, 2013, 496, 343-346] and even gave insight into the structural dynamics during a chemical reaction [Jean-Ruel, H. et al., J. Phys. Chem. A, 2011, 115, 13158-13168]. The main challenge for first applications of FED on proteins is sufficient sample preparation and development. Currently samples need to be single-crystalline, reversible and match a size of about 100-150 nm thickness combined with a lateral size on the order of 100 μm x 100 μm. The presented work will outline and discuss current approaches for sample development on model systems Bacteriorhodopsin and give an overview on the femtosecond electron diffraction method including the limitations for samples. Latest results on a crystalline organic spin-crossover system [Jiang, Y., Ultrafast Phenomena XIX, 2014, in press] as well as on the model system Bacteriorhodopsin are reported to demonstrate the intrinsic capabilities of FED.

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Development of Cryo-Electron Microscopy Sample Preparation for the Examination of Nanobubble

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Cryo-electron microscopy (cryo-EM) has been widely used to characterize bio-macromolecules, such as DNA molecules, proteoliposomes, and protein complexes. The targets of interests were effectively remained the original structure and morphology in amorphous ice. However, in cryo-EM, target of interests in gas phase has not been studied yet. In this study, we utilized cryo-EM to examine the nanobubbles in frozen aqueous solution with a ''sandwich'' TEM grids assembly. With significantly increased surface area and lower buoyancy, nanobubble system greatly enhances the efficiency of oxygenation and the time of retention in medical application. The substrate of ''sandwich'' assembly provides sufficient nucleation site for the formation of bubbles. Additionally, elimination of water-air interface increases the possibility to capture bubbles in nano scale. This method demonstrates a promising way to evaluate bubble system using cryo-EM and provides an insight on the study of nanobubble in biomedical application.

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Time-Resolved cryo-EM Study of Ribosome Subunit Association by Mixing-Spraying

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Time-resolved cryogenic electron microscopy (cryo-EM) is a technique for visualizing transient structures in a biological specimen in a pre-equilibrium system. Capturing reactions in the sub-second range has been a practical challenge, due to the requirement of depositing the specimen rapidly on the grid without blotting. To capture faster reactions in the sub-second range, Lu and coworkers [1] developed a mixing-spraying method, which allows a reaction involving two macromolecular components to proceed for tens to hundreds of milliseconds. The first study of ribosome subunit association using time-resolved cryo-EM, by Shaikh et al. [2], were performed at 9.4 ms and 43 ms. In present work, we improved the mixing-spraying method, by designing an environmental chamber and optimizing the EM data yield, and applied the method to the study of ribosome subunit association. We captured the subunit association reaction in a pre-equilibrium state, by mixing the subunits and reacting for 60 ms and 140 ms. Our results showed that at 60 ms and 140 ms time points, 33% and 42% of the large subunits have associated into 70S ribosomes, respectively, compared with 85% in a 15-min incubation control experiment. Three distinct conformations of the 70S ribosome were found: non-rotated, non-rotated with 30S head swiveled and rotated. Our results demonstrate the capability of the mixing-spraying method of time-resolve cryo-EM to visualize multiple states of macromolecules in a reaction within a sub-second time frame. In the future, the mixing-spraying method will be applied to study translation initiation, to gain insights on the role of mRNA, initiator tRNA and initiation factors.

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A Computational Modeling of Macromolecular Ensemble Conformation and Blurring in Cryo EM

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It is well known that Cryo EM results in 3D construction in which subnuits of a macromolecular complex may appear to be blurred and bloated. This issue affects the accuracy of positional and orientational information about the subnits extracted from such reconstructions. The blurring can be due to classification or averaging steps in the analysis or due to mobility of the quaternary macromolecular structure. Such effects cannot be captured by traditional models of EM. In this paper we propose a mathematical framework and algorithm to model this phenomenon. We use clustering methods such as the K-means algorithm to roughly resolve the different rigid subunits in the 3D density obtained from a macromolecular complex. Then we model the blurring effect in each (resolved) submit as an ensemble of rigid body motions (i.e., orientations and translations), specifically we use the Gaussian probability density (with unknown covariance and mean) on the group of rigid body motions in 3D. We relate the shape of each resolved (blurred) subunit to the unknown parameters of the Gaussian using the known structure of the subnit (from PDB). This gives a system of equations with unknowns being the parameters of the Gaussian. The system, in general, is underdetermined, however, we impose physically meaningful regularization constraints to obtain unique solutions. Thereby we are able to obtain more accurate orientation and positional information. We show the performance of the algorithm on simulated data. We also discuss prospect of this method in combination (or fusion) with SAXS data in order to reinforce information from both modalities.

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A Computational Modeling of Macromolecular Assemblies in SAXS

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The determination of the structure of large macromolecular complexes is essential to reveal the function of the complexes in biological setting. For that purpose, several experimental methods for the structural determination have been developed and applied such as Electron Microscopy (EM) and Small-Angle X-ray Scattering (SAXS). In this study, we focus on SAXS. In particular, we are interested in determining the spatial relationship between rigid subunits in a given macromolecular complex, which is especially important because conformational dynamics of rigid components in the given complex is directly related to its function. The present work is to develop a more efficient and effective computational framework to reveal spatial relationship between complex subunits. One of important quantities in SAXS includes the so-called distance distribution function (or pair distribution function), which is the distribution of distance between every pair of points in the complex. Given rigid subcomponents of the complex, this important quantity can be determined more efficiently when we apply the mathematical concept of the Fourier transform for rigid-body motion group. To this end, we develop a mathematical model to calculate the pair distribution function for a complex structure consisting of several rigid sub-components. This new model is verified with several examples. In the end, our modeling efforts will combine the current methodology with similar ones for other experimental methods (e.g. EM) to reveal more refined biological macromolecular structures.

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Sub-Surface Serial Block Face Scanning Electron Microscopy

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Serial block face scanning electron microscopy (SBF-SEM) provides nanoscale 3D ultrastructure of tissue samples up to several hundred micrometers in size. In SBF-SEM, an ultramicrotome built into the SEM specimen stage successively removes thin sections from a plastic-embedded, heavy metal-stained specimen. After each cut, the freshly exposed block face is imaged at a low incident electron energy using the backscattered electron signal, which is sensitive to heavy atoms in the sample. Although the x-y resolution in the plane of

the block face is approximately 5 nm, the resolution along the z-axis in SBF-SEM is limited by the minimum slice thickness of around 25 nm. We have explored the feasibility of improving the z-resolution in SBF-SEM by recording images at more than one primary beam energy, thus sampling different depths below the block surface. We used Monte Carlo simulations of SEM images from an epoxy block containing 5-nm diameter carbon spheres stained with 14% osmium positioned at different depths, as a model for small biological structures. A linear relationship was found between the depth of the spheres and the ratio of backscattered signals at primary beam energies of 1.4 keV and 6.8 keV, which allowed us to generate 3D tomograms with a depth resolution of around 5 nm. Experiments are in progress to test this technique using a Zeiss Sigma-VP SEM equipped with a Gatan 3View SBF system. Sub-surface SBF-SEM could potentially match focused ion beam (FIB) SEM in terms of z-resolution, but with the added advantage of providing higher throughput and larger tissue volumes. The research was supported by the intramural program of NIBIB.

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Fixed Path Length Sample Holders Enable Robust Cryosaxs Measurements from Sub-Microliter Sample Volumes

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Small angle x-ray scattering (SAXS) gives structural information about biological molecules in solution. However, large (~30 microliter) sample volumes are needed to mitigate radiation damage, limiting the use of SAXS in studying rare molecules. By cryocooling SAXS samples, radiation damage and required sample volumes are reduced by orders of magnitude [1], but challenges in creating identically-sized frozen samples complicate background subtraction. Here we present microfabricated silicon sample holders for cryoSAXS. These rigid sample holders have a fixed x-ray path length, simplifying background subtraction. Less than 800 nL of sample are required, facilitating measurements on expensive or hard-to-express molecules. These fixed path length, low volume sample holders make cryoSAXS a more accessible technique capable of probing a wide range of biological molecules.

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3D Dynamical Observations of Single Molecule Motions by X-Rays, Electron and Neutron

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We have proposed that single molecule techniques using shorten wavelengths, for example, X-rays, electrons, and neutron [1]. Especially, Diffracted X-Ray Tracking (DXT) using normal synchrotron orbital radiation (SR) source (not XFEL) has been developed for obtaining the information of the 3D internal motions of single proteins with both high time-resolution (micro-seconds) and high precision (nm/1000) [2, 3]. DXT can be monitored through trajectories of the Laue diffraction spots from the nanocrystal which was labeled on the individual proteins. This concept can apply to utilize by using both electrons and neutron. Instead of the Laue diffraction using white X-ray, the Electron Back-Scattered Diffraction Pattern was adopted to monitor the 3D orientations of the nanocrystals linked to the single protein molecules[4]. We called Diffracted Electron Tracking (DET). Additionally, we call Diffracted Neutron Tracking (DNT) for new single molecule measuring method in which the long time observation from the non-destructivity of a neutron is possible.

DXT, DET and DNT are assigned to labeling techniques through the nanocrystals. The size effect between intramolecular motions of individual proteins and the labeled nanocrystals becomes very important. We succeeded in the analysis of the quantitive size effects. As a result, we pointed out a possibility that determinations of the intramolecular motions without labeled nanocrystals are carried out quantitatively. Additionally, by progressing of the automatic DXT analysis corresponding to huge diffraction information, we obtained the time-resolved dynamical information that statistical reliability is sufficiently high.

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Accurate Determination of Tautomeric/Protonation States in Quantum-Mechanic Driven Macromolecular Crystallographic Refinement

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Structure Based Drug Discovery (SBDD) is employed by virtually all pharmaceutical research and development organizations. Gaining an understanding of the protein:ligand complex structure along with the proper protonation and explicit solvent effects is crucial for obtaining meaningful results from docking, thermodynamic calculations, active site exploration, and ultimately lead optimization. Recently, we incorporated our linear-scaling, quantum mechanics (QM) DivCon tool with Phenix (e.g. Phenix/DivCon) in order to accurately elucidate the protein:ligand complex molecular structure. An intrinsic problem of the X-ray crystallographic data is its inability to detect hydrogen atoms - even at higher resolutions. It is generally extremely difficult to experimentally determine the protonation/tautomeric state of the ligand and the surrounding active site. Traditionally, protonation can be established using the neutron diffraction; however, experimental requirements such as reliance on very large crystals and on deuterium exchange limit the method's suitability in SBDD.

In order to address this X-day data deficiency, we have challenged Phenix/DivCon with various protonation candidates and applied rigorous statistical analyses to measure the agreement between the 3D structure of each candidate with electron density. While through the experiment we still cannot directly observe hydrogen atoms, using the accurate QM functional we are able to observe the presence/absence of hydrogen atoms by studying their influences on bound heavy atoms (Carbon, Nitrogen, Oxygen). To evaluate our protocol we have chosen two protein:ligand structures 4N9S and 2JJJ for which both neutron and X-ray structures and data are available in PDB. Ten probable protonation states for the ligands in those structures have been generated, and each of the possible candidates has been refined against X-ray data with Phenix/DivCon. We have found out that the top scored tautomer in each case coincides with the ligand structure revealed by the neutron diffraction.

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Transmission X-Ray Imaging Detector Captures the Last Light at NSLS

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Biological research constitutes a large and expanding scientific focus at synchrotron facilities. Structural biology researchers using x-ray facilities make up the majority of this community, including use of techniques such as macromolecular x-ray crystallography, small-angle x-ray solution scattering, x-ray microscopy, x-ray absorption spectroscopy, x-ray fluorescence and x-ray footprinting. Many of these technologies, as they are developed to take advantage of next-generation synchrotron sources, are trending toward use of high flux beams and/or beams which require enhanced stability and precise understanding of beam position and intensity from the front end of the beamline all the way to the sample. For high flux beams, major challenges include heat load management in optics (including the vacuum windows) and a mechanism of real-time volumetric measurement of beam properties such as flux, position, and morphology. For beam stability in these environments, feedback from such measurements directly to control systems for optical elements or to sample positioning stages would be invaluable. For x-ray footprinting, a focused ''white beam'' is used to maximize x-ray flux density over a practical sample size using a toroidal mirror. This intense beam can melt beryllium windows and is very complicated to measure, causing difficulties with properly focusing the mirror and with understanding where the beam is and exactly what is being delivered to the sample. To address these challenges, we are developing diamond-based instrumented vacuum windows with integrated volumetric x-ray intensity, beam profile and beam-position monitoring capabilities. The prototype device will be used as the exit window for the XFP beamline currently being developed at NSLS-II for x-ray footprinting. Current progress is presented, including successful demonstration of a >1kilopixel free-standing transmission imaging detector that was used to capture the last x-ray photons at the National Synchrotron Light Source.